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Fermentation kinetics makeover in poly-ε-lysine biosynthesis by *Streptomyces noursei* NRRL 5126

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Abstract

An unstructured model has been used to predict microbial growth based on glycerol consumption and poly-ε-lysine (ε-PL) biosynthesis by *Streptomyces noursei* NRRL 5126. The logistic and Luedeking-Piret equations have been proposed to describe the time course of ε-PL formation, substrate consumption and cell growth. The shake flask level data from kinetic studies was illustrated and compared with fermenter studies. In all cases, the model simulation matched well with the experimental observations, which made it possible to elucidate the fermentation characteristics of *Streptomyces noursei* during efficient ε-PL production from glycerol. Optimized oxygen supply into the fermenter studies shifted mixed growth associated biosynthesis of ε-PL from shake flask level to growth associated biosynthesis.

Keywords: Fermentation, Kinetics, Luedeking-Piret eqation, Poly-ε-lysine, *Streptomyces noursei*

Introduction

Poly-ε-lysine (ε-PL) is an unusual, naturally occurring homopolyamide of $_L$-lysine, having amide linkage between ε-amino and α-carboxyl groups. It is biodegradable, edible and non-toxic towards human and environment. Potential applications of ε-PL and its derivative have been of multifarious interest in a broad range of industrial fields like foods, pharmaceuticals and medicine (Shih et al. 2006). ε-PL is mainly used as a natural food additive (Hiraki 2000) and its safety has been confirmed by *in vivo* studies (Neda et al. 1999).

ε-PL is produced industrially by aerobic fermentation of a filamentous bacterium, *Streptomyces albulus* (Shima and Sakai 1977). *Streptomyces noursei* has some advantages over *S. albulus* with respect to pH dependency for growth and ε-PL formation.

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Although the yields of ε-PL are lower with *S. noursei*, optimization of fermentation parameters and strain improvement by conventional and molecular techniques could improve it to the levels produced by *S. albulus* (Bankar and Singhal 2010). Substantially, no intensive study has yet been reported on the biosynthesis of ε-PL with *S. noursei*.

The development of kinetic models is necessary for understanding, controlling, and optimizing fermentation processes. Kinetic modeling is regarded as an indispensable step in developing a fermentation process, since the models can be used to determine an optimal operation condition for the production of a target metabolite (Garcia-Ochoa et al. 1990).

The rational design and optimization of industrial fermentations requires an understanding of production kinetics. Several unstructured kinetic models have been proposed to describe the fermentative production of biopolymers such as xanthan (Garcia-Ochoa et al. 1990), gellan (Wang et al. 2006) and hyaluronic acid (Huang et al. 2007). The most widely used unstructured models for describing cell growth are the Monod kinetic model, the logistic equation and the Haldane model. A useful analytical model for biopolymer fermentation kinetics includes temporal variations of substrate (S), biomass (X) and biopolymer products (P).

Microbial growth: The logistic equation

In certain cases of biopolymer fermentation, the microbial processes do not follow the classical kinetic model of substrate-limited biomass growth and product formation proposed by Monod. Therefore, the logistic equation, a substrate-independent model, is used as an alternative empirical function (Luedeking and Piret 1959). In many fermentation systems, cell growth has been characterized by the logistic equation. The logistic equation (equation 1) can be described as follows:

$$\frac{dx}{dt} = \mu_m \left(1 - \frac{X}{X_m}\right) \times X \qquad (1)$$

where μ_m is the maximum specific growth rate (1/h) and X_m is the maximum attainable biomass concentration (g/l). The integrated form of equation 1 using $X=X_0$ ($t = 0$) gives a sigmoid variation of X as a function of t, which may represent both an exponential and a stationary phase The usual approach for logistic equation is based

on formulation in which specific growth rate is related to the amount of unused carrying capacity (*k*):

$$X=\frac{X_0 e^{kt}}{1-\frac{X_0}{X_m}\left(1-e^{kt}\right)} \qquad (2)$$

k can be estimated from the log-log plot of $\frac{1}{X}\cdot\frac{dX}{dt}$ and $1-\frac{X_0}{X_m}$ as an intercept (Shuler and Kargi 2002).

Product formation: Luedeking-Piret equation

The production of many microbial metabolites follows the classical equation of Luedeking and Piret (1959). According to this model, the product formation rate (r_P) depends on both the instantaneous biomass concentration and the growth rate in a linear manner.

$$r_p=\alpha . r_x+\beta . X \qquad (3)$$

where r_p and r_x are the rates of the formation of P and X, respectively; α and β are the parameters determined experimentally, that provides the framework for classifying microbial metabolites into primary (β = 0), secondary (α = 0), and mixed (α ≠ 0 and β ≠ 0).

The integration of above equation 3 (where $P = P_o$ at $t = 0$) with equation 2 yields:

$$P_{(t)}=P_o+\alpha X_o\left(\frac{e^{kt}}{1-(X_o/X_\infty)(1-e^{kt})}-1\right)+\beta\frac{X_m}{\mu_m}\ln\left(1-\frac{X_o}{X_m}(1-e^{kt})\right) \qquad (4)$$

The equation can be used to determine the concentration of product formed at time t during the fermentation. The model employs rate equations for biomass (*X*), the ε-PL biosynthesis (*P*) and the glycerol utilization (*S*) to describe the fermentation process. Equation 3 can be changed to as follows:

$$P=\alpha . X+\beta X . t+P_0+\Phi \qquad (5)$$

Where Φ is the product of α and X_0 in numerical value

Glycerol uptake: the modified Luedeking-Piret equation

Glycerol is used in present study to form cell components and metabolic products as well as for the maintenance of cells. The consumption of glycerol can be explaied by modified Luedeking-Piret equation (equation 6)

$$-\frac{ds}{dt}=\left(\frac{1}{Y_{x/s}}\right)\frac{ds}{dt}+\left(\frac{1}{Y_{p/s}}\right)\frac{dp}{dt}+m_s X \qquad (6)$$

where $Y_{x/s}$ is the cell yield coefficient for glycerol, $Y_{p/s}$ is the product yield coefficient for glycerol, and m_s is the maintenance coefficient (g substrate/g cell.h).

Combining equations 3 and 6 gives:

$$-\frac{ds}{dt}=\left(\frac{1}{Y_{x/s}}+\frac{\alpha}{Y_{p/s}}\right)\frac{dx}{dt}+\left(\frac{\beta}{Y_{p/s}}+m_s\right)X \qquad (7)$$

Glycerol consumption can be expressed by simplified form of equation 6 as:

$$-\frac{ds}{dt}=\gamma\frac{dX}{dt}+\delta . X \qquad (8)$$

where γ is the sum of $1/Y_{X/S}$ and $\alpha/Y_{P/S}$ and δ is the sum of $\beta/Y_{P/S}$ and m_s. Similarly, the integration of equation 8 with $S=S_0$ at t = 0 yields:

$$S_{(t)}=S_0-\gamma . X_0\left\{\frac{e^{kt}}{\left[1-(X_0/X_m)(1-e^{kt})\right]}-1\right\}-\delta\frac{X_m}{\mu_m}\ln\left[1-\frac{X_0}{X_m}(1-e^{kt})\right]$$

This equation can be used to determine unutilized glycerol in the medium.

Data processing

Fits of the model to the data were performed by linear regression by using the least-squares method, which commonly involves the implicit assumption that the distribution of errors is normal:

$$\sum_{i=1}^{n}\left(y_i-\hat{y}\right) \qquad (9)$$

where $\tilde{y}_i$ is the mean value.

The performance of the models was evaluated by using correlation coefficients (*r*):

$$r=\sqrt{1-\frac{RSS}{\sum_{i=1}^{n}(y_i-\hat{y}_i)^2}} \qquad (10)$$

Where RSS is residual of square, *n* is the number of data points, y_i is the observed value, and $\tilde{y}_i$ is the fitted value.

To the best of our knowledge, no investigations have been reported on the kinetic studies on fermentative production of ε-PL. The present study aims to fit a suitable model to deduce the proportion of substrate consumption and/or product formations related to biosynthesis and cell growth from *S. noursei* NRRL 5126, and compare the model with experimental observations from shake flask level and from fermenter (5 L).

Materials and methods

The chemicals and media components used in present study were of analytical grade and purchased from Hi-Media Limited, Mumbai, India. ε-PL was obtained as a gift sample from Handary Bio-Engineering B.V. Netherlands.

Fermentations

Bacterial strain, *S. noursei* NRRL 5126 was a gift sample from ARS Culture Collection, USA. It was maintained on a medium containing (g/l) glucose 4, yeast extract 4, malt extract 10 and agar 15, pH 7.2-7.4. For production, medium previously optimized in our laboratory containing (g/l) proteose peptone 10, glycerol 30, $(NH_4)_2SO_4$ 8, $FeSO_4•7H_2O$ 0.03, $MgSO_4•7H_2O$ 0.5, $ZnSO_4•7H_2O$ 0.04, K_2HPO_4 0.8 and KH_2PO_4 1.36 was used. Citric acid and aspartic acid were added after 24h and 36h fermentation with 5mM and 2mM respectively (Bankar and Singhal 2010; 2011a). The initial pH of the medium was adjusted to 6.8 with 1 M NaOH before autoclaving at 121 °C for 20 min. The flasks were incubated for 120 h on a rotary shaker at 30±2 °C and 180 rpm. All experiments were carried out at least in triplicate.

Cell growth and ε-PL production in fermenter

Seed medium (50 ml) was inoculated with 1 ml (3.8×10^6 cells/ml) of cell suspension prepared with cells from a 5-day-old culture in

saline and incubated at 30±2 °C for 48 h with shaking at 180 rpm. This inoculum was used for production of ε-PL in 5 L stirred tank bioreactor, BIOSTAT Bplus (Sartorius, Germany) with 2 L working volume equipped with a pH probe (Type InPro 3030; Mettler Toledo) and a dissolved oxygen (DO) probe (Hamilton, switzerland). Fermenter vessel (UniVesses) was offering a height-to-diameter ratio of 2:1 with an internal concave bottom for optimum mixing. It was equipped with four baffles with six-flat blade disc impellers (two) measuring 64 mm diameter, spaced 9 cm from each other. 4% (8.3×10^8 cells/ml) pre-cultured seed of *S. noursei* NRRL 5126 was inoculated into 2 L sterile fermentation medium and then cultured for 120 h at 30±2 °C and maintained using a re-circulating water bath (Bankar and Singhal 2011b). During the fermentation, the pH, DO and aeration (gas flow) was monitored on line while, ε-PL titer, cell growth, and residual glycerol was analyzed off-line by the methods seen in the analytical method section.

Analysis of ε-PL

The culture broth was centrifuged (8000g, 10 min), and the ε-PL concentration was measured in the supernatant using the method of Shen et al. (1984). Briefly, 1 ml of supernatant was mixed with trypan blue solution and absorbance was measured at 580 nm on Helios (α) UV-VIS spectrophotometer (Thermo Electron, Germany).

Dry cell weight determination (DCW)

The relationship between the optical density at 660 nm and the DCW (70°C till constant weight) was established with initial experiments, and the resulting equation was used to determine the DCW for further experiments by measuring the optical density after suitable dilutions.

Glycerol estimation

Glycerol in the fermentation broth was estimated by a colorimetric procedure described by Bok and Demain (1977). To 1 ml fermentation broth, 1 ml of 0.015 M sodium metaperiodate in 0.12M HCl and 2 ml of 0.1 % rhamnose was added. Addition of 4 ml Nash reagent produced a yellow colored product after keeping the reaction mixture on water bath at 53°C which had a strong absorbance at 412 nm.

Results and discussion

Kinetic analysis of microbial growth in batch cultivation

Batch fermentation by *S. noursei* NRRL 5126 showed a classical growth trend. Cell growth remained constant after 96 h and reached its maximum value of 10.39 g/l and 18.76 g/l after 120 h in shake flask and fermenter level respectively. In contrast, the maximum value of specific growth rate was observed at the beginning of the process and then declined to approximately zero after 108 h for both shake flask level and fermenter level. A logistic equation was used to express the cell growth and specific cell growth as shown in Fig. 1 and Fig. 2.

The model predictions for cell growth and specific growth rate were basically consistent with the experimental results from the exponential phase to the stationary phase (correlation coefficients > 0.955), which demonstrated that this model was suitable for prediction of growth of *S. noursei* NRRL 5126 under the given conditions. The lower value of coefficient of variation as 0.6 indicates a greater reliability of the experimental performance.

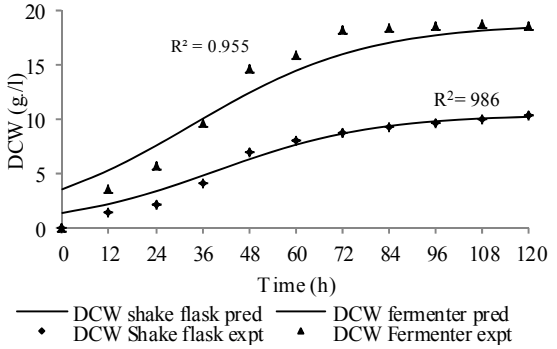

Figure 1. The comparison of experimental data and calculated values of DCW for shake flask and fermenter studies with *S. noursei* NRRL 5126.

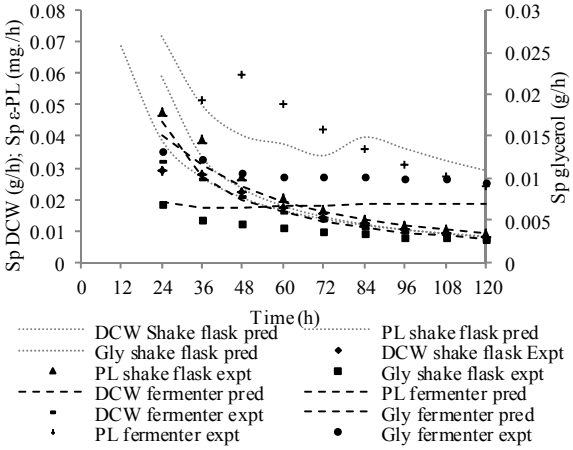

Figure 2. Comparison between experimental results and the model prediction for specific growth rate, specific product formation rate and specific substrate consumption rate in shake flask and fermenter studies with *S. noursei* NRRL 5126.

Kinetic analysis of product formation in batch cultivation

The kinetics of product formation was based on the Luedeking-Piret equation. The growth associated parameter α, calculated for the production of ε-PL showed different values at shake flask (3.79 mg.ε-PL/g.cell) and fermenter level (42.11 mg ε-PL/g.cell) (Table 1). During the fermenter studies, optimized parameters of aeration and agitation favored the microbial growth as compared to shake flask level and ultimately increased the ε-PL yield as seen from Fig.1 and Fig. 2 (Bankar and Singhal 2011b). The non growth associated parameter β, calculated for the production of ε-PL for shake flask (1.20 mg ε-PL/g.cell.h) and fermenter level (1.78 mg ε-PL/g.cell.h) were found to be constant.

In shake flask level, the close α and β value shows significant effect and clearly reveals that the ε-PL production is mixed growth associated and increases after increase in cell growth after 36 h throughout the exponential growth phase until the stationary phase. The larger value of α in the fermenter level proved growth associated biosynthesis of ε-PL. The ratio of α to β at shake flask level equals 3.18 and which confirms that there is mixed growth associated product formation, while at fermenter level, the value representing ratio of α to β was 23.66 and confirmed growth associated production of ε-PL. This shift of mixed growth associated biosynthesis from shake flask level to growth associated biosynthesis of fermenter level is probably due to the change in

operational conditions, especially oxygen concentration in fermenter level. Our previous studies revealed higher oxygen supply at fermenter level to result in higher cell mass formation and ε-PL biosynthesis as compared to shake flask level (Bankar and Singhal 2011b).

The sudden decrease in ε-PL production after 108 h in the culture medium at shake flask level was presumably caused by digestion with a peptide hydrolase(s) produced by ε-PL producer which act as self protector for microorganism (Saimura et al. 2008). Although an experimental evaluation for ε-PL degrading enzyme was not performed, the production of this enzyme in *S. noursei* NRRL 5126 appeared to be low at fermenter level which resulted in less degradation of the ε-PL in the fermentation broth as seen from the Fig. 3. A comparison of model predicted and experimental values for ε-PL production are given in Fig. 3. After fitting the experimental data to eq. (5), the following equation was used to describe the relationship between ε-PL formation and cell concentration:

Shake flask level $P = 3.79.X + 1.19.\ X.t + 8.417$
Fermenter level $P = 42.11.X + 1.78.\ X.t + 175.38$

A comparison between model predictions and the experimental data for specific product formation rate is shown in Fig. 2.

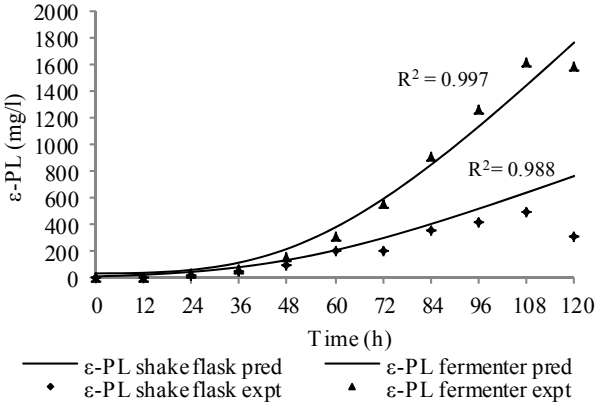

Figure 3. The comparison of experimental data and calculated values of ε-PL formation for shake flask and fermenter studies with *S. noursei* NRRL 5126.

Kinetic analysis of substrate consumption in batch cultivation

In ε-PL fermentation, an increase in biomass concentration was accompanied by a gradual decrease in residual glycerol concentration. Consumption of glycerol is represented in equation 8. The rate of glycerol utilization was almost similar at shake flask level and fermenter level with slightly higher consumption rate at fermenter level (Fig. 4). Glycerol concentration decreased rapidly from its initial value of 3% after 36 h of fermentation and gradually decreased thereafter till the end of fermentation (120 h) (Bankar and Singhal 2011b). This model is very suitable for describing glycerol consumption and specific glycerol consumption (correlation coefficients > 0.94). The values for γ and δ for shake flask level and fermenter level with modified equation are shown in Table 1.

Conclusions

Controlled aeration and agitation rates significantly influenced the production of ε-PL with *S. noursei* NRRL 5126 which shifted mixed growth associated biosynthesis of ε-PL from shake flask level to growth associated biosynthesis at fermenter level. Kinetic and mathematical parameters were successfully employed for the prediction of data with respect to time.

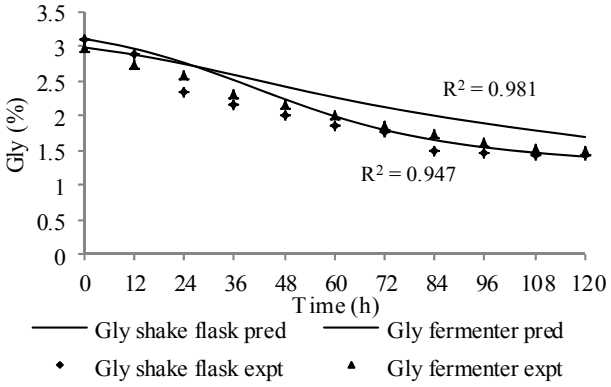

Figure 4. The comparison of experimental data and calculated values of glycerol consumption for shake flask and fermenter studies with *S. noursei* NRRL 5126.

Table 1 Estimation and comparison of the model parameters from experimental data for shake flask and fermenter level

Parameters	Shake flask studies	Fermenter studies
μ_m (1/h)	0.056	0.052
α (ε-PL/g)	3.79	42.11
β (ε-PL/g.h)	1.20	1.78
γ (g/g)	0.167	0.048
δ (g/g.h)	3×10^{-4}	4.35×10^{-4}
k (g/l)	0.049	0.045
$Y_{x/s}$(g/g)	6.17	11.911
$Y_{p/s}$(ε-PL/g)	297.29	1111.512
$Y_{p/x}$(ε-PL/g)	50.167	86.504
m (g/g.h)	0.032	0.0060
Productivity (mg/l.h)	4.61	15.026

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